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Novel Patterns of Amino Acid Mutations in the Hepatitis B Virus Polymerase in Association With Resistance to Lamivudine Therapy in Japanese Patients With Chronic Hepatitis B

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Lamivudine is effective in suppressing replication of hepatitis B virus (HBV). However, the emergence of HBV variants resistant to lamivudine is a concern. Lamivudine resistance has been attributed mainly to a substitution of isoleucine or valine for methionine at residue 550 (M550I or M550V) in the catalytic site of the virus polymerase. A substitution of methionine for leucine at residue 526 (L526M) has also been identified. To examine such virus genotypic mutations in Japanese patients, we studied five patients with chronic hepatitis B, who showed HBV breakthrough while on a 1-year lamivudine treatment. The entire nucleotide and deduced amino acid sequences of the proposed reverse transcriptase domain of the polymerase gene were determined on HBV DNA amplified by polymerase chain reaction from patient sera collected at the start and at the end of therapy. The HBV sequences from all five patients were of genotype C. In four patients, a substitution of valine or isoleucine for leucine at residue 426, which has not been reported previously, emerged in combination with M550I. One also harbored L526M. In the remaining patient, an alteration of leucine to methionine at residue 428 co-occurred with M550V. Longitudinal study of the mutations showed that the two or three mutations in each patient emerged almost simultaneously 4 weeks before or at the time of breakthrough and were replaced by wild-type virus after completing the therapy. Our results indicate that occurrence of HBV polymerase mutations at residue 426 in combination with M550I is frequent in Japanese or genotype C virus-infected patients who develop resistance to lamivudine. ***J. Med. Virol. 59:270–276, 1999.***

KEY WORDS: HBV DNA; reverse transcriptase; YMDD motif; HBV breakthrough; HIV

INTRODUCTION

Persistent hepatitis B virus (HBV) infection can result in serious liver diseases with eventual progression to cirrhosis or development of hepatocellular carcinoma. Interferon has been licensed for the treatment of patients with chronic HBV infection, but beneficial responses have been observed in only about one-third of patients [Lok et al., 1993; Wong et al., 1993; Lau et al., 1997]. Recently, lamivudine (2′, 3′-dideoxy-3′-thiacytidine), a deoxycytidine analogue, has been shown to inhibit HBV replication in vitro [Doong et al., 1991; Chang et al., 1992a, 1992b] and in vivo [Dienstag et al., 1991]. Lamivudine treatment for HBV-infected patients has been shown to reduce serum virus DNA levels rapidly below the detection limit of standard assays and to have few adverse side effects [Schalm et al., 1995; Bain et al., 1996; Benhamou et al., 1996; Grellier et al., 1996; Ben-Ari et al., 1997; Nevens et al., 1997; Nery et al., 1998]. Lamivudine treatment has also been shown to reduce hepatic necroinflammatory activity [Honkoop et al., 1997; Lai et al., 1998]. However, long-term lamivudine therapy can induce the emergence of drug-resistant HBV strains in some patients receiving liver transplantation [Ling et al., 1996; Tipples et al., 1996; Bartholomew et al., 1997; Allen et al., 1998] or with chronic hepatitis [Allen et al., 1998; Buti et al., 1998; Chayama et al., 1998; Lai et al., 1998; Niesters et al., 1998]. The HBV strains isolated from patients exhibiting an incomplete response or virus breakthrough while on lamivudine therapy have been reported to harbor a substitution of isoleucine or valine for methionine at residue 550 (M550I or M550V) within a highly conserved tyrosine-methionine-aspartate-aspartate

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(YMDD) nucleotide binding site of the polymerase (The residue positions in the polymerase were counted according to HBV genome units of genotype C [Norder et al., 1993; Ogata et al., 1993]). Among several other mutations, a substitution of methionine for leucine at residue 526 (L526M) has been reported to occur in association with the M550V mutation [Ling et al., 1996; Bartholomew et al., 1997; Allen et al., 1998; Buti et al., 1998; Niesters et al., 1998]. Thus, accumulating evidence indicates that specific amino acid substitutions in the HBV polymerase render the virus resistant to lamivudine. However, the number of patients and the extent of the gene region that have been examined so far are limited.

We examined five Japanese patients with chronic hepatitis B, who exhibited virus breakthrough associated with relapses in serum aminotransferase values while on lamivudine therapy, for the occurrence of amino acid mutations in the HBV polymerase by determining entire amino acid sequence of the proposed reverse transcriptase (RT) domain. The results demonstrated novel patterns of residue mutations that may be related to HBV resistance to lamivudine.

METHODS

Patients

Patients in this study were some of those who participated in the Phase III lamivudine clinical trial (Glaxo-Wellcome, Stevenage, UK) undertaken in Japan, in which oral administration of 100 mg per day of lamivudine was given for 1 year (52 weeks). The trial of 19 biopsy-proven chronic hepatitis B patients was carried out at Niigata University Hospital, Niigata, Japan, from June 1995 to March 1997. Eligibility criteria included a positive test for serum HBV DNA by the branched DNA signal amplification (bDNA) assay (Chiron Corp., Emeryville, CA) of which the detection limit was 0.7×10^6 (0.7 mega) genome equivalents per milliliter (0.7 Meq/mL) and elevated values of alanine aminotransferase (ALT) greater than twice the upper limit of normal range, which was 40 international units per liter (IU/L), within 3 months before the start of therapy. Patients were excluded if they had signs of decompensated liver disease. All patients were negative for hepatitis delta virus, hepatitis C virus, or human immunodeficiency virus (HIV). No patient had received treatment with antiviral, immunomodulatory, or corticosteroid therapy for at least 6 months before starting the therapy. Among the 19 patients, 5 were selected for this study on the basis of a breakthrough of virus replication while on lamivudine treatment. Breakthrough patients were defined as those with an initial response leading to undetectable serum levels of HBV DNA measured by bDNA assay that later during treatment became detectable again.

The study was conducted according to the Declaration of Helsinki and Good Clinical Practice. The Ethics Committee for Human Experimentation of Niigata University Hospital approved the protocol. Every patient gave written informed consent.

Laboratory Tests

Patients were monitored every 4 weeks during the therapy by biochemical liver function tests, serum HBV surface antigen (HBsAg) and HBV e antigen/antibody (HBeAg/anti-HBe) enzyme immunoassays (Abbott Laboratories, Abbott Park, IL), and serum HBV- bDNA assay.

A portion of serum obtained from the patients was stored at −80°C until HBV DNA extraction.

Polymerase Chain Reactions and Sequencing

Extraction of HBV DNA from serum samples and polymerase chain reactions (PCR) were carried out essentially as described previously [Ogata et al., 1997]. In brief, 100 μl of serum was digested in a lysis buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM ethylenediamine tetraacetic acid (EDTA), 50 μg/ml proteinase K, and 0.1% (wt./vol.) sodium dodecyl sulfate at 37°C for 3 hr, followed by phenol/chloroform extraction and ethanol precipitation. The DNA samples were dissolved in distilled water and prepared for the first round of PCR in a reaction volume of 100 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 mM each dNTP, 0.1 μM sense and antisense primers, and 2.5 units of *Taq* DNA polymerase (Perkin Elmer Japan-Applied Biosystems, Chiba, Japan). A thermal cycle of 94°C for 1 min, 45°C for 1 min, and 72°C for 2 min was repeated for 35 cycles. The second round of PCR was carried out under the same conditions as the first PCR by using 10 μl of the first PCR solution. Two sets of nested primer pairs were prepared to amplify the whole of the predicted RT domain of HBV polymerase gene [Radziwill et al., 1990]. One primer set that covered the spacer domain and around two-thirds of the RT domain was as follows: outer primers were 5′CACCTGCAGCCTCATTTTGTGGGTCACCATA3′ and 5′CATAAGCTTCACAAGTCTCTGACATACTTTCCAAT3′, and inner primers were 5′GTGCTGCAGTTTGTGGGTCACCATATTCTTG3′ and 5′GACAAGCTTTTGACATACTTTCCAATCAATAG3′. Another primer set that covered around one-third of the RT domain and the RNase H domain was as follows: outer primers were 5′ATTCTGCAGTTTCTTTTGTCTTTGGGTAT3′ and 5′CCAAAGCTTCCCAAGGCACAGCTTGGAGGCTT3′, and inner primers were 5′AATCTGCAGCTTTTGTCTTTGGGTATACATTT3′, and 5′GCCAAGCTTGGCACAGCTTGGAGGCTTGAACAG3′. (Underlined nucleotides denote restriction enzyme recognition sites for *Pst*I or *Hin*dIII introduced into the primers.)

The PCR-amplified HBV DNA was subjected directly to nucleotide sequencing. When necessary, the PCR products were cloned into the plasmid vector pUC18 or pUC19 (Takara Biochemicals, Shiga, Japan) and 6–10 clones were sequenced. The sequencing reactions for both directions were carried out using a dye terminator cycle sequencing kit (Perkin Elmer Japan-Applied Biosystems). The primer used in each sequencing reaction

TABLE I. Patient Characteristics and Responses to Lamivudine

					At the start of therapy			At the end of therapy		
Patient	Age (years)	Gender	HBV subtype	HBV genotype	HBeAg/ anti-HBe	Serum HBV DNA (Meq/mL)	Serum ALT (IU/L)	HBeAg/ anti-HBe	Serum HBV DNA (Meq/mL)	Serum ALT (IU/L)
A	35	Male	*adr*	C	(+)/(−)	258	73	(+)/(−)	119	79
B	48	Male	*adr*	C	(+)/(−)	1300	108	(+)/(−)	220	97
C	45	Male	*adr*	C	(+)/(−)	185	149	(+)/(−)	14	71
D	39	Male	*adr*	C	(+)/(−)	221	71	(+)/(−)	2600	233
E	35	Male	*adr*	C	(+)/(−)	5.7	51	(+)/(−)	2.2	50

HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase.

was either the internal primer for the second round of PCR or one synthesized based on the HBV sequence. The reaction products were run on an automated DNA sequencer (ABI PRISM Model 377, Perkin Elmer Japan-Applied Biosystems). Thus, nucleotide sequences that covered a whole gene region of the predicted RT domain of the HBV polymerase gene, that is, nucleotides (nt) 135–1166, which corresponded to the polymerase residues 348–691, were determined.

The subtype and genotype of the infected HBV were determined on the basis of amino acid sequence of the major surface (S) protein, which was deduced from the nucleotide sequence of the corresponding gene region.

RESULTS

Baseline Characteristics of Patients and Kinetics of Serum HBV DNA Levels While on Lamivudine Therapy

Baseline characteristics of the five patients are summarized in Table I. All five patients were infected with HBV of subtype *adr* and genotype C. Table I also summarizes the serum HBe status, HBV DNA levels, and ALT values at the start and at the end of 52 weeks of lamivudine therapy. All patients were positive for serum HBeAg throughout the course of therapy. Serum ALT values in patients A, D, and E exceeded 80 IU/L at least once during the 3 months before initiating therapy.

The kinetics of HBV DNA levels in serum measured by bDNA assay during and after therapy in each patient is shown in Figure 1. All patients showed successive time points with negative tests for serum HBV DNA after initiating the therapy, but all of them became positive for the virus DNA again after week 32 on the therapy. Thus, all five patients showed breakthrough virus.

Sequence Analysis of the RT Domain of the HBV Polymerase Gene

First, the entire nucleotide and deduced amino acid sequences of the proposed HBV RT domain were compared for each patient between the beginning of therapy (week 0 of therapy) and the end of therapy (week 52 of therapy). The comparison confirmed earlier reports that at the end of therapy every patient with HBV breakthrough carried virus mutants with an altered residue in the YMDD locus. The virus from patients A, B, C, and D had T instead of G at nt 743, generating M550I, and that the virus from patient E possessed G instead of A at nt 741, introducing M550V (Fig. 2). The comparison also identified a C to A mutation at nt 669, causing the substitution L526M in the virus from patient D. In addition to these mutations frequently identified in earlier reports, one other HBV mutation was found in every patient with the M550I HBV mutant. The virus from these patients had G in patient A or A in patients B, C, and D instead of C at nt 369, generating a valine or isoleucine substitution for leucine at residue 426 (L426V or L426I). The virus from patient E with the M550V variation had a substitution of A for T at nt 375, introducing an alteration from leucine to methionine at residue 428 (L428M) (Fig. 2). By sequencing clones constructed from the PCR products, we confirmed that nucleotide substitutions that generated the two or three residue changes in each patient occurred in the same HBV DNA molecules.

Next, a longitudinal examination was undertaken for the appearance of these HBV mutations in each patient. The results demonstrated that the two or three residue substitutions in each patient took place almost simultaneously. The substitutions appeared 4 weeks before or at the time of the virus breakthrough, were retained until the end of therapy, and were replaced by wild-type virus after ceasing the therapy (Fig. 2). In patient D, M550I was detected one time point (4 weeks) earlier than other residue mutations. In patient C, the reversion occurred 20 weeks after completing the therapy.

Direct sequencing data from the initial detection of mutation at nt 369, which was responsible for the L426V/I variation in the virus from patients A, B, C, and D, indicated the presence of a mixed population of two mutant nucleotides, A and G (Fig. 3a). With the progress of therapy, the mixed population changed to a pure nucleotide population of G in patient A (Fig. 3b) or a pure nucleotide population of A in patients B, C, and D (Figs. 3c and 3d). Nucleotide A at nt 375 at L428M in patient E did not show signals for the coexistence of other mutant nucleotides (Fig. 3e). Such nucleotide populations, mixed or pure, at given positions were also confirmed by sequencing 6–10 clones obtained from the PCR products.

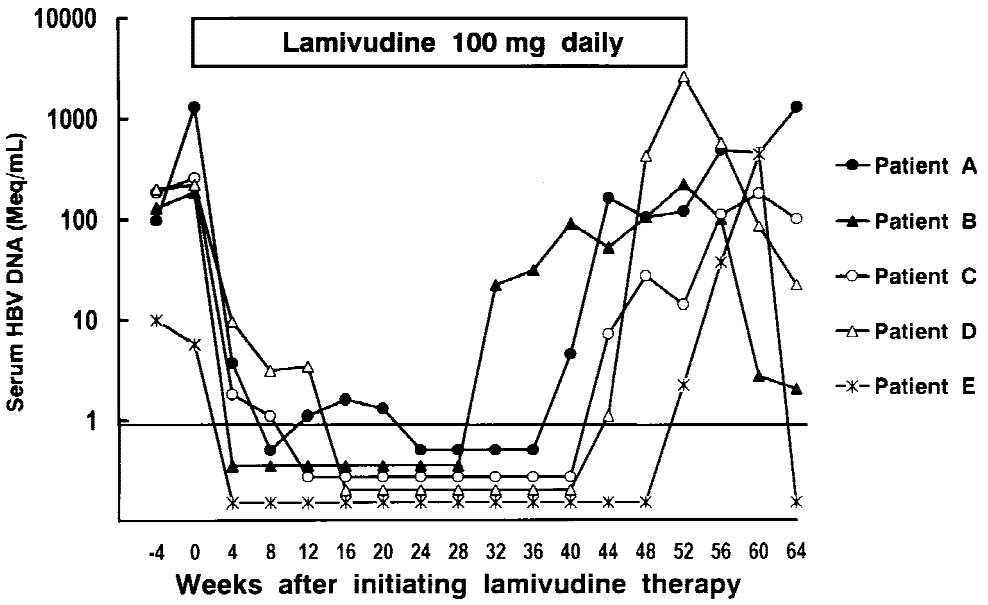

Fig. 1. Serum hepatitis B virus (HBV) DNA levels before, during, and after lamivudine treatment in five chronic hepatitis B patients with virus breakthrough while on lamivudine treatment. The virus DNA levels were measured by the branched DNA amplification assay. A horizontal line denotes the detection limits of the DNA levels of 0.7×10^6 (0.7 mega) genome equivalents per milliliter (Meq/mL).

		Nucleotide number																				P gene codon position																			
	Genogroup C Consensus	369					375					669					741		743			426		428		526		550													
		TGG	C	TA	TCG		C	TG		CTC	CTG					TAT	ATG		GAT	GAT		W	L	S	L	L	L	Y	M	D	D										
Patient	Weeks on therapy																																								
A	0	---	-	-	-----		T	--		---	---					---	---		---	---		-	-	-	-	-	-	-	-	-	-										
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	32	---	-	-	-----		T	--		---	---					---	---		---	---		-	-	-	-	-	-	-	-	-	-										
	36	---	G/A	-	-----		T	--		---	---					---	--T		---	---		-	V/I	-	-	-	-	-	I	-	-										
	*40	---	G	-	-----		T	--		---	---					---	--T		---	---		-	V	-	-	-	-	-	I	-	-										
	52	---	G	-	-----		T	--		---	---					---	--T		---	---		-	V	-	-	-	-	-	I	-	-										
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	28	---	G/A	-	-----		T	--		---	---					---	--T		---	---		-	V/I	-	-	-	-	-	I	-	-										
	*32	---	A	-	-----		T	--		---	---					---	--T		---	---		-	I	-	-	-	-	-	I	-	-										
	52	---	A	-	-----		T	--		---	---					---	--T		---	---		-	I	-	-	-	-	-	I	-	-										
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	52	---	A	-	-----		-	--		---	A--					---	--T		---	---		-	I	-	-	-	M	-	I	-	-										
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	48	---	-	-	-----		A/T	--		---	---					---	G--		---	---		-	-	-	M/-	-	-	-	V	-	-										
	*52	---	-	-	-----		A	--		---	---					---	G--		---	---		-	-	-	M	-	-	-	V	-	-										
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Fig. 2. Longitudinal study of nucleotide and predicted amino acid changes in the putative reverse transcriptase domain from breakthrough hepatitis B virus (HBV) of lamivudine-treated patients. Consensus sequences of nucleotides and predicted amino acids in the genogroup C are shown on the top line. Nucleotide numbers are counted by defining the second nucleotide in the hypothetical *Eco*RI site in the HBV genome as number one. The residue positions of the polymerase were counted according to HBV genome units of genotype C. Dashes (–) denote nucleotides or amino acids identical to the consensus sequences. The week on therapy with an asterisk (*) indicates a time point when the virus breakthrough occurred.

DISCUSSION

The occurrence of amino acid mutations of HBV polymerase was studied in five Japanese patients with chronic hepatitis B, who exhibited virus breakthrough while on a 1-year lamivudine treatment.

It was found that the L426V/I mutations occurred in

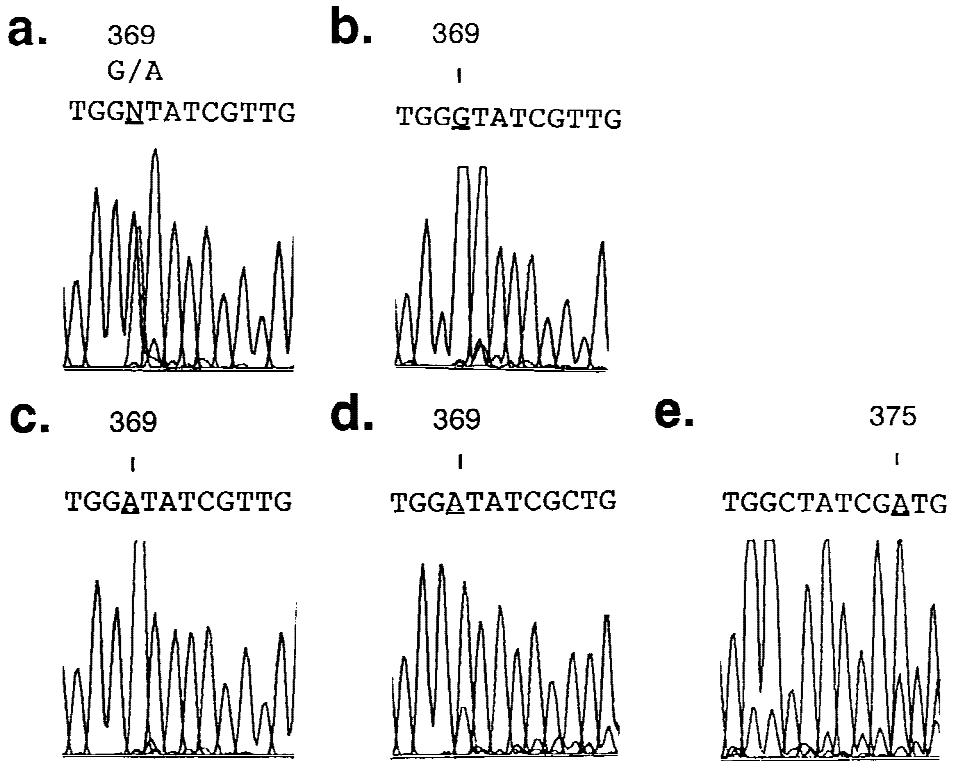

Fig. 3. Representative fragments of the nucleotide sequence at nt 366–377 of hepatitis B virus genome containing nucleotide substitutions at nt 369 and 375, which caused residue mutations L426V/I and L428M, respectively. (**a**) Nucleotide sequence from patient A at week 36 on therapy; (**b**) nucleotide sequence from patient A at week 40 on therapy; (**c**) nucleotide sequence from patient B at week 32 on therapy; (**d**) nucleotide sequence from patient C at week 52 on therapy; (**e**) nucleotide sequence from patient E at week 52 on therapy. Nucleotides underlined denote substitutions from wild-type nucleotides while on lamivudine treatment.

combination with the M550I mutation in four patients and the L428M mutation emerged in association with the M550V mutation in the remaining patient. To our knowledge, the L426V/I mutations have not been reported previously, whereas L428M has been found in only one patient with M550I [Allen et al., 1998]. Although the L526M mutation has been observed almost consistently with M550V [Ling et al., 1996; Bartholomew et al., 1997; Allen et al., 1998; Buti et al., 1998; Niesters et al., 1998], this mutation was found with M550I in a patient. Coexistence of L526M and M550I has also been reported in one Japanese patient [Chayama et al., 1998]. The reasons for such differences in the mutational patterns of the polymerase residues in relation to lamivudine resistance are unknown. One possible explanation may be differences in the genotypes of the infecting virus. All five patients studied were infected with the genotype C-HBV, which is endemic in Asia, whereas most viruses examined previously for the gene region that encompassed residues 426 and 428 were probably from Europe or North America [Allen et al., 1998; Niesters et al., 1998], where the genotype A- or D-HBV is prevalent [Norder et al., 1993]. In this context, the polymerase residue mutation at 426 may be frequent in Japanese or genogroup C-HBV infected patients who show resistance to lamivudine treatment.

The importance of the L426V/I and the L428M mutations for inducing virus resistance to lamivudine or changing virus biology is unknown at present. Indeed, in vitro studies using genetically engineered mutant HBV genomes have demonstrated that HBV constructs containing M550I/V are less sensitive to lamivudine [Allen et al., 1998; Fu and Cheng, 1998; Ladner et al., 1998; Melegari et al., 1998] and replicate less well [Allen et al., 1998; Fu and Cheng, 1998; Ladner et al., 1998] as compared with wild-type virus. Such studies have also shown that an addition of L526M [Allen et al., 1998; Fu and Cheng, 1998; Melegari et al., 1998] or one other mutated residue [Fu and Cheng, 1998] to virus constructs carrying M550I/V alters drug sensitivity or replication ability of the YMDD variants of HBV. Our results from observation of the clinical kinetics of the mutant viruses in each patient showed almost simultaneous turnover of L426V/I with M550I in patients A, B, C, and D and L526M in patient D or of L428M with M550V in patient E. These findings suggest that L426V/I and L428M were involved in the virus resistance to lamivudine in combination with the YMDD-mutations and L526M. In addition, results from serum virus levels before, during, and after the therapy in each patient indicated that serum levels of HBV variants with L426V/I and M550I in patients A, B, and C or with L428M and M550V in patient E were lower than those of wild-type virus, whereas serum levels of the variant with L426V/I, L526M, and M550I in

patient D were higher than wild-type virus. These findings may support the results from in vitro studies that mutational patterns of the polymerase residues can affect replication ability of the YMDD mutants of HBV, although roles of L426V/I or L428M in the virus replication are unclear because we did not find HBV with mutations only in the YMDD motif. Studies in vitro or in vivo on HBV variants carrying L426V/I or L428M would clarify biological significance of these mutations.

Lamivudine-resistant variants of HBV emerge less frequently and less rapidly than those of HIV during the therapy [Schuurman et al., 1995]. This difference could be attributed to a lower mutation rate of hepadnaviruses [Girones and Miller, 1989] than that of HIV and to constraints due to the overlapping open reading frame of the S gene on the polymerase. However, because nucleotide substitutions that generated L426V/I and L428M did not confer changes in the major S protein, the S gene constraints could not account for the simultaneous emergence of these mutated residues with M550I/V. Nevertheless, nucleotide substitutions for the L426V/I and the L428M were all transversions, that is, substitutions between a purine and a pyrimidine. This is a rare substitution event [Gojobori et al., 1982; Keulen et al., 1996], suggesting a selection and a fitness of these residue mutations while undergoing lamivudine therapy. According to a recently proposed three-dimensional model of the HBV RT [Allen et al., 1998], the residues 426 and 428, which are only two residues apart, both would reside on the beta strand adjacent to the YMDD loop, suggesting some interactions of these two residues with lamivudine.

Thus, clinical kinetics, types of nucleotide substitutions, and possible locations in the RT of L426V/I and L428M suggest possible roles of these residue mutations in the virus resistance to lamivudine possibly in combination with the YMDD-mutations.

Because we did not evaluate liver histology during or after lamivudine therapy, it is not known whether the pathogenicity of the mutant viruses we identified is increased or reduced as compared with wild-type virus. However, elevation of serum aminotransferase values after the HBV breakthrough would suggest that changes of therapy from lamivudine monotherapy are preferable when lamivudine-resistant mutants emerge. Combination therapy with interferon [Schiff et al., 1998] may be a choice if clinical trials of such therapies are proved to be more effective than lamivudine monotherapy. Also, a nucleotide analogue adefovir is reported to be active against lamivudine-resistant variants with M550I, with M550V, or with L526M and M550V [Xiong et al., 1998].

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